OIPE IAP47
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PATENT & TRADEMARK OFFICE

AMENDMENTS TO THE SPECIFICATION:

Amend the specification as follows:

Page 3, delete the paragraph spanning lines 24-25 and insert the following therefor:

Figure 1 sets out Table 1, providing the coordinates of a RAD51-BRCA2 BRC4 complex structure (SEQ ID NO:1 is the BRC4 sequence and SEQ ID NO:2 is the consensus sequence),

Page 5, delete the paragraph spanning lines 1-19 and insert the following therefor:

Figure 7 shows (a) a superposition of the RAD51-BRCA2 complex on a subunit of the crystallographic RecA filament (omitting RAD51 for clarity), the BRC motif being positioned at the interface between adjacent RecA subunits in the filament, (b) a close view of part of the interface between subunits in the crystallographic RecA filament, the sequence 26-IMRL-29 in the amino terminal tail of RecA mediating polymerisation by antiparallel beta strand pairing, and residues Ile26 and Leu29 representing points of hydrophobic contacts between subunits, (c) a close view of part of the interface between RAD51 and the BRC motif, the BRCA2 sequence 1524-FHTA-1527 (amino acids 1-4 of SEQ ID NO:16) interacting with RAD51 via antiparallel beta strand pairing, and residues Phe1524 and Ala1527 contacting RAD51 hydrophobically, and (d) a demonstration of evolutionary conservation of RAD51 residues predicted to be involved in nucleoprotein filament formation, sequences of human DMC1, pyrococcus (an archea

bacterium) RADA, bacterial RecA and human BRCA2 with a comparable structural role being aligned underneath, and RAD51 residues completely or highly conserved being boxed (SEQ ID NO:3 is RAD51 of H. sapiens, C. griseus and X. laevis, SEQ ID NO:4 is RAD51 of D. melanogaster, SEQ ID NO:5 is RAD51 of S. cerevisiae, SEQ ID NO:6 is DMC1 of H. sapiens, SEQ ID NO:7 is RADA of P. furiosus, SEQ ID NO:8 is RecA of E. coli and SEQ ID NO:9 is BRCA2 BRC4 of H. sapiens), and

Page 6, delete the paragraph spanning lines 20-24 and insert the following new paragraph therefor:

A flexible polypeptide linker (such as $(Gly)_{12}$ (SEQ ID NO:10), $(Ser)_{12}$ (SEQ ID NO:11), or $(GlySer)_{6}$ (SEQ ID NO:12)) may be used to join the RAD51 and the BRC repeat sequence. Preferably the linker allows substantially unrestrained interaction between the BRC repeat sequence and the RAD51.

Page 12, delete the paragraph spanning lines 7-11 and insert the following new paragraph therefor:

The formation of such mutants is described in the accompanying examples. The mutant may be formed by substitution, deletion and/or addition of at least one amino acid in the 85-GFTTATE-91 (SEQ ID NO:3) sequence of human RAD51, or the corresponding sequence in other forms of RAD51.

Page 12, delete the paragraph spanning lines 16-21 and insert the following new paragraph therefor:

Preferably the mutation substantially alters the functionality of the sequence. For example, in the accompanying examples we replaced the hydrophobic residue Phe86 or Ala89 in the 85-GFTTATE-91 <u>(SEQ ID NO:3)</u> sequence of human RAD51 with hydrophilic glutamic acid. Other suitable mutations would be apparent to the skilled person.

Please delete the paragraph spanning line 29 of page 21 through line 13 of page 22 and insert the following new paragraph therefor:

We show in the examples below that the BRC repeats encoded in BRCA2 structurally mimic a sequence in RecA that contributes to the interface between successive subunits in the RecA filament, and we present evidence that RAD51 multimerization in nucleoprotein filament formation proceeds through a similar interface. The sequence 85-GFTTATE-91 <u>(SEQ ID NO:3)</u> in RAD51 closely resembles the conserved BRC repeat sequence (GFxTASG) <u>(SEQ ID NO:13)</u> that mimics RecA. Furthermore, replacement of Phe86 or Ala89 in RAD51 with glutamic acid, predicted to disrupt critical hydrophobic contacts, creates mutants that are no longer capable of filament formation when expressed in mammalian cells. Thus, our findings uncover an evolutionarily conserved structural motif that enables RecA and RAD51 to assemble into multimeric filaments essential for DNA recombination, and that has become incorporated into BRCA2, a protein exclusive to higher eukaryotes.

Please delete the paragraph spanning line 27 of page 31 through line 15 of page 32 and insert the following new paragraph therefor:

In order to favour BRCA2 binding over RAD51 multimerisation, we covalently joined the BRC repeat to RAD51. The BRCA2 BRC type 4 sequence (amino acids 1517 to 1551) was connected to the amino terminus of a RAD51 sequence spanning the RecA homology domain (Ser97 to the natural carboxyl terminus) via the flexible polypeptide linker: (ThrGlySer)$_4$MetGly (SEQ ID NO:14), designed to allow for unrestrained interaction between the BRC repeat sequence and RAD51. The chimaeric protein was expressed in *E. coli* fused to a double amino-terminal tag consisting of a six histidine sequence followed by a GST tag. The soluble, overexpressed protein was first purified from the crude bacterial lysate by Ni-NTA agarose chromatography. The tag was cleaved by incubation with TEV protease and removed by glutathione agarose chromatography. The protein was purified to homogeneity by two further steps of anion exchange chromatography on a ResourceQ column and gel filtration on a Superdex200 10.30 HR column (Amersham-Pharmacia). The protein was concentrated to 12 mg/ml (0.36micromolar), flash frozen in liquid nitrogen and stored in aliquots at –80° C.

Please delete the paragraph spanning line 27 of page 31 through line 15 of page 32 and insert the following new paragraph therefor:

BRC4 remains in continuous contact with the ATPase domain of RAD51 over a sequence stretch of 28 amino acids (Leu1521 to Glu1548), defining a minimal BRC

repeat footprint on RAD51 (Figure 4). Residues Phe1524 to Val1532 fold into a beta hairpin with a 3:5 loop (1526-TASGK-1530) <u>(SEQ ID NO:15)</u> structured as a type I turn followed by a beta bulge at residue Gly1529, which has a positive ϕ torsion angle [19]. The hairpin lines up alongside beta strand B3, thereby extending RAD51's beta sheet by two short anti-parallel strands. After the hairpin, the BRC motif wraps around helix A4 of RAD51 by means of a short linker (residues Lys1533 to Ala1535) that kinks abruptly at residue Lys1536 and leads into an amphipathic alpha-helical segment (residues Lys1536 to Val1542). The remaining residues at the carboxyl end of BRC4 (residues Val1542 to Glu1548) form an irregular coil with elements of a 3_{10} helix, that spans helices A4 and A5 of RAD51, making an angle of 60° to their axes. Altogether, the BRC motif encircles approximately a third of the hypothetical circumference of RAD51 at its point of maximum diameter.

Page 38, delete the paragraph spanning lines 5-24 and insert the following new paragraph therefor:

Residues 1524-FHTASGK-1530 <u>(SEQ ID NO:16)</u>, with the exception of His1525, form a contiguous block of highly conserved amino acids. Phe1524 is the single most conserved BRC residue (present in 89% of the sequences in a set of 56 BRC repeats from seven different organisms): the structure shows that it is involved in a crucial recognition interaction with RAD51. Thr1526 does not contact RAD51, but accepts a hydrogen bond from the main chain nitrogen of Lys1530 that is essential for the conformation of the 3:5 hairpin loop. Thr1526 also donates a hydrogen bond to the hydroxyl function of Ser1528, thus keeping it poised for interaction with RAD51 Asp187.

The amino acids threonine or serine account for 93% of occurrences at this position. Like Phe1524, Ala1527 (conserved in 82% of BRC repeats) provides another important point of hydrophobic contact with RAD51. Ser1528 (59%) and Lys1530 (79% preference for a basic residue) are engaged in a polar interaction with Asp187 of RAD51. The preference for a glycine, serine or asparagine (combined frequency of 93%) at position 1529 is dictated by the conformational requirement for a residue that can tolerate a positive ϕ torsion angle.

Please delete the paragraph spanning line 16 of page 40 through line 4 of page 41 and insert the following new paragraph therefor:

The structure of BRCA2-bound RAD51 reveals some unexpected features of its nucleotide-binding site (see Figures 6(a) and (b)). Lys133 and Thr134, in Walker motif A (127-GEFRTGKT-134) <u>(SEQ ID NO:17)</u>, and Asp222, in Walker motif B (218-LLIVD-222) <u>(SEQ ID NO:18)</u>, are sequestered in a solvent-inaccessible hydrogen-bonding network that extends to Tyr159, Asp161 and Thr165 via a buried water molecule (Figure 6(a)). Exposed Phe129 at the tip of the phosphate-binding loop (P-loop or Walker motif A) buries part of its aromatic ring in a hydrophobic interaction with Thr134 and Thr165. These contacts do not take place in RecA [18,20], because Lys72 and Thr73 of motif A are further apart from Asp144 in motif B, whereas Glu68 replaces Phe129 in the P-loop. Possibly reflecting the presence of this additional set of interactions, the overall conformation of the P-loop is different in RAD51. A 3-D superposition (Figure 6(b)) shows that, whereas the P-loop remains unchanged in the apo- and ADP-bound forms of RecA [18,20], in BRCA2-bound RAD51 it adopts a more closed conformation that is

unlikely to be compatible with its occupation by the ATP phosphates. Although the BRC repeat does not directly mask the ATP-binding site, we speculate that it may cause an indirect conformational effect when bound to RAD51 that inhibits ATP binding.

Please delete the paragraph spanning line 21 of page 41 through line 8 of page 42 and insert the following new paragraph therefor:

The structural basis for filament formation by RAD51 is not known [23,24]. In order to gain an insight into the mechanism deployed by BRCA2 to regulate RAD51 filament formation, we analysed the RAD51-BRCA2 interaction in the context of the crystallographic RecA filament (see Figures 7(a) to (d)). In the crystal [18], the RecA molecules pack into a spiral that resembles the nucleoprotein filament formed *in vivo*. Overlaying the RAD51-BRCA2 complex on RecA results in the localization of the BRC beta hairpin at the interface between two adjacent RecA molecules [18] within the crystallographic filament (Figure 7(a)). Surprisingly, BRC4 residues 1523-GFHTASG-1529 <u>(SEQ ID NO:9)</u> superimpose closely onto the RecA sequence 25-SIMRLGE-31, which is part of the interface between RecA subunits. RecA residues 27-MRL-29 add in fact an anti-parallel beta strand to the central beta sheet of a neighbouring RecA molecule, in an identical fashion to the interaction of BRC4 residues 1525-HTA-1527 with RAD51 in the RAD51-BRCA2 complex (see Figures 7(b) and (c)). Moreover, RecA residues Ile26 and Leu29 make comparable hydrophobic contacts to those made by Phe1524 and Ala1527 of BRC4 with RAD51.

Page 42, delete the paragraph spanning lines 9-23 and insert the following new paragraph therefor:

The superposition analysis provides a strong clue concerning the mechanism adopted by BRCA2 to regulate RAD51 function - BRCA2 binding prevents formation of the nucleoprotein filament by interfering with a crucial contact between RAD51 subunits, and the specific role of the BRC repeats is to mimic the conformation of the RAD51 segment involved in such contact. One prediction of our proposed mechanism is that sequence similarity should be found between the BRC motif and the region of the RAD51 sequence with a putative role in multimerization analogous to that performed by RecA sequence 25-SIMRLGE-31 (SEQ ID NO:8). Indeed, careful inspection of the RAD51 sequence for short motifs resembling the BRC consensus GFxTASG (SEQ ID NO:13) motif identifies the highly conserved sequence 85-GFTTATE-91 (SEQ ID NO:3) in the RAD51 linker between the amino terminal domain and the catalytic core (Figure 7(d)).

Page 42, delete the paragraph spanning lines 24-26 and insert the following new paragraph therefor:

To test the proposed mechanism, we constructed mutant RAD51 molecules in which amino acids Phe86 and Ala89 within the sequence 85-GFTTATE-91 (SEQ ID NO:3) were replaced by glutamic acid.

Please delete the paragraph spanning line 30 of page 43 through line 6 of page 44 and insert the following new paragraph therefor:

Based on our crystallographic and biological data we therefore conclude that the RAD51 sequence 85-GFTTATE-91 (SEQ ID NO:3) forms an essential part of the interface between RAD51 monomers in the nucleoprotein filament, and residues Phe86 and Ala98 constitute essential points of hydrophobic contact. The sequences 85-GFTTATE-91 (SEQ ID NO:3) in RAD51 and 25-SIMRLGE-31 (SEQ ID NO:8) in RecA mediate a mode of association between subunits that represent a common structural feature of their nucleoprotein filaments.

Page 44, delete the paragraph spanning lines 7-15 and insert the following new paragraph therefor:

We further conclude that BRCA2 blocks nucleoprotein filament formation by binding to RAD51 with the BRC consensus sequence GFxTASG (SEQ ID NO:13), which structurally mimics the RAD51 sequence 85-GFTTATE-91 (SEQ ID NO:3). In the RAD51-BRC4 complex, BRC4 residues Phe1524 and Ala1527 play the same roles that RAD51 residues Phe86 and Ala89 have in the association between RAD51 monomers. The interaction surface between RAD51 and the BRC repeat is more extensive than that provided by the GFxTASG (SEQ ID NO:13)sequence only, as would be expected for a dominant antagonist interaction.

Please delete the paragraph spanning line 17 of page 44 through line 6 of page 45 and insert the following new paragraph therefor:

Point mutations affecting conserved residues within the BRC repeats predicted to be important for RAD51 binding occur in patients who develop familial breast cancer (Breast Cancer Information Core database, accessible at [[http:://
www.]]the following URL: nhgri.nih.gov/Intramural_research/Lab_transfer/Bic/). The common cancer-associated Thr1526 -> Ala mutation impairs the ability of a BRCA4 peptide to bind RAD51 [7,17]. The structure shows that formation of a hydrogen bond between the hydroxyl function of Thr1526 and the main chain nitrogen of Lys1530 is critical to the conformational integrity of the BRC hairpin loop (Figure 5b). The mutation therefore impairs the affinity of BRCA2 to RAD51 by destabilizing the conformation of the beta hairpin that apposes the BRC repeat to the surface of RAD51. Consistent with the notion that the hydroxyl function mediates an essential interaction, position 1526 is occupied by either a threonine or a serine in 52 out of 56 BRC repeat sequences from seven different organisms (Table 3). BRC repeats in which the threonine is replaced are unlikely to assume the 3:5 hairpin loop conformation required for efficient binding to RAD51. Loss of the critical hydroxyl function at a position analogous to that occupied by Thr1526 in BRC4 has been noted in breast cancer-associated mutations that affect BRC1 (Thr1012 -> Arg) or BRC7 (Thr1981 -> Ile).

Insert the attached Sequence Listing after the figures.